**Determination of methyl order parameters   
using solid state NMR under off magic angle spinning**

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**Abstract**

Quantification of dipolar couplings in biological solids is important for the understanding of dynamic processes. Under Magic Angle Spinning (MAS), order parameters are normally obtained by recoupling of anisotropic interactions involving the application of radio frequency pulses. We have recently shown that amide backbone order parameters can be estimated accurately in a spin-echo experiment in case the rotor spinning angle is slightly mis-calibrated. In this work, we apply this method to determine methyl order parameters in a deuterated sample of the SH3 domain of chicken α-spectrin in which the methyl containing side chains valine and leucine are selectively protonated.

**Keywords:** solid-state NMR, off Magic Angle spinning, Spin Echo, Microcrystalline proteins, perdeuteration

**Introduction**

Many biomolecular processes such as molecular recognition, enzyme catalysis and ligand binding require a certain amount of flexibility or dynamics in the structure. Initially, solution-state NMR and later solid-state NMR has provided powerful methods to characterize such processes in biomolecules.

Dennis Torchia pioneered this work with experiments on the characterization of dynamics of staphylococcal nuclease (Cole et al. 1988; Cole and Torchia 1991). The theoretical analysis of the 15N longitudinal relaxation data for various jump and diffusive motional models is based on the seminal paper by Torchia and Szabo (Torchia and Szabo 1982). These experiments were inspiration for a whole generation of scientists. With the advent of higher magnetic fields, faster spinning, availability of high quality samples and atom-specific assignments in the solid-state, these experiments have been applied to uniformly isotopically enriched proteins to measure backbone amide (Giraud et al. 2004) (Chevelkov et al. 2008), as well as for carbon 13Cα relaxation rates in a proton and carbon dilute environment (Asami et al. 2015). Application of model-free (Lipari and Szabo 1982) and extended model-free (Clore et al. 1990) approaches resulted in a quantification of dynamic processes for micro-crystalline proteins (Chevelkov et al. 2009b; Schanda et al. 2010), as well as for HET-s prion fibrils (Smith et al. 2016). Torchia and co-workers compared structure and dynamics of staphylococcal nuclease in solution and in the crystal (Cole et al. 1988). This analysis became a prototypic study for later investigations in which the dynamic properties of proteins in liquid and solid phase were compared (Reif et al. 2006) (Chevelkov et al. 2010). The analysis of dynamics of keratin and collagen fibrils carried out by the Torchia lab had an important impact on the understanding of the mechanical properties of this protein material (Batchelder et al. 1982; Jelinski et al. 1980; Mack et al. 2002), and is a role model how solid-state NMR allows to better understand motional processes in biology. The analysis of deuterium line shapes of alanine methyl deuterated collagen samples (Jelinski et al. 1980) showed how the collagen molecules in the collagen fibrils reorient. The motional model is extracted from the measured order and asymmetry parameters. Today, experiments are carried out at ever increasing MAS rotation frequencies, and new methods are needed to extract information on order and asymmetry parameters under these conditions.

Under magic angle spinning, dipolar interactions are suppressed and need to be reintroduced by application of a multiple-pulse scheme. In the past, phase inverted CP (CPPI) (Chevelkov et al. 2009a), REDOR-type experiments (Schanda et al. 2010), DIPSHIFT (Munowitz et al. 1981) or its phase-alternating R-symmetry (PARS) based analogues (Hou et al. 2014), as well as cross polarization with variable contact time (CP-VC) (Paluch et al. 2015) have been used to measure these one-bond dipolar interactions accurately. We have recently shown that amide backbone order parameters can be quantified using off-magic angle spinning (Xue et al. 2019). We show here that this method is applicable for the determination of order parameters in a heavily deuterated sample of the SH3 domain of chicken α-spectrin. For the experiments, samples are prepared in which the methyl groups are selectively protonated in the γ1 position for valines as 13CHD2, and at the δ1 position for leucines, using a specifically methyl-labeled acetolactate precursor (Gans et al. 2010). In this work, we have measured the 13C-1H dipolar coupling of methyl groups in valine and leucine in heavily deuterated SH3 domain protein using slightly off magic angle sample spinning condition. The methyl groups are labelled as 13CHD2 in 100% of the γ1 sites in valine and 100% of the δ1 sites in leucine. Comparison of the obtained results with the order parameters obtained in REDOR type experiments (Asami and Reif 2013) shows that offMAS and REDOR methods yield very similar values for the extracted order parameter.

**Results and Discussion**

High resolution NMR experiments for spinning solids traditionally require that the rotor is oriented at an angle () of 54.74° () with respect to B0. Under this condition, the components of the second rank anisotropic interactions such as dipolar interaction and chemical shift anisotropy (CSA) vanish (Andrew et al. 1958, 1959). As a result, only the isotropic interactions such as isotropic chemical shifts and scalar couplings are retained in the spectra. In case distances between two nuclei or chemical shift tensors are to be quantified, RF pulses need to be employed to recouple the anisotropic interactions. In the past, robust recoupling methods have been developed to enable the quantification of dipolar coupling or CSA (Levitt 2007).

The RF powers needed to recouple anisotropic interactions are typically on the order of 3-5 times the MAS frequency, with a few exceptions where RF requirements are less severe. For MAS frequencies ranging between 60-110 kHz, the RF powers thus exceed the practical limits. Fast MAS (> 60 kHz) in combination with high B0 fields turned out to yield excellent spectral quality in biological samples. At the same time, the need for large amounts of samples decreases: Even though the rotor volume becomes smaller, sensitivity is not compromised. This makes fast MAS an attractive tool to study biological samples.

We have recently shown that performing NMR experiments under a slightly mis-adjusted rotation angle (henceforth referred to as OffMAS), allows accurate determination of 15N-1H order parameters (Xue et al. 2019) in a protein sample. First implemented by the Levitt group to measure dipolar coupling in homonuclear systems for small molecules, this method is applicable at an arbitrary MAS frequency and has no restrictions with respect to the necessary RF power. In this experiment, in-phase 15Nx/y magnetization is monitored as a function of the spin-echo delay using a single π refocussing pulse (Figure 1). The echo modulation curve of the powder averaged signal under off-MAS can be written as (Pileio et al. 2007; Pileio et al. 2008; Sarkar et al. 2015):

[Eq. 1]

The spin echo signal in Eq.1 consists of two components. The second part is dependent on the crystallite orientations, therefore affected by powder averaging of spin echo signals. By contrast, the first part is independent of crystallite orientations. The parameter *p* determines the relative contributions of the signal components. and represent the decay constant of the first and second spin echo signal components, respectively. The oscillation of the spin echo signal is governed by , which is defined as:

[Eq. 2]

is a function of the 1H,13C heteronuclear scalar coupling (*J*) and dipolar coupling defined as . The analytical solution of Eq.2 is (Pileio et al. 2007):

[Eq. 3]

Where

When , *i.e*, the rotor angle is set to the magic angle, the spin echo signal is modulated by the *J* coupling. A positive deviation of from zero leads to a faster oscillation whereas a negative leads to a slower oscillation compared to a pure *J* oscillation. This additional oscillation under offMAS is proportional to the product of the dipolar coupling *b* and as described by Eq. 2.

The spin echo curve is fitted to Eq. 2 using six parameters (*p*, *J*, , *b*, *T*20and *T*2J), where (*p*, *T*20 and *T*2J) are empirical parameters, which govern the decay of the spin echo curves. On the other hand, (*J*, , *b*) determine the frequency of the spin echo oscillations.



***Figure 1:*** *Pulse sequence used to record methyl 13C-1H dipolar couplings in methyl groups of valine and leucine in the αSH3 domain. The phases were cycled as follows: φ1= x, -x; φ2= x, x, -x, -x; φrec= (x, -x, -x, x), (-x, x, x, -x). All other phases were kept along x, unless otherwise specified. 13 echo delays up to 32 ms were recorded in an interleaved way.*

In comparison to the amide order parameter experiment described previously, certain peculiarities have to be taken into account for the quantification of methyl order parameters. The rigid limit for the 15N-1H dipolar coupling is 11478 Hz. The dipolar coupling for a 1H, 13C spin-pair in a methyl group amounts to 21794 Hz assuming a bond-length of 1.115 Å. Due to the rapid rotation of the methyl group on the ps timescale, the dipolar coupling values are scaled down to 7265 Hz. Since the scaling occurs along the C3 axis, the dipolar coupling tensor can be assumed to be axially symmetric. In comparison to the amide case, experiments are recorded at a larger accounting for the smaller effective dipolar coupling of a methyl group. In the fit of the OffMAS data, *b* and appear as one single parameter (*b*\*). Therefore, the value of was determined using the spin echo for L10δ1 using a 1H, 13C dipolar coupling value of 5040 Hz.



***Figure 2:*** *(A) Representative 2D 1H,13C correlation spectra obtained from the pseudo 3D OffMAS experiment at echo delays of 200 μs, 5.8 ms and 32.2 ms, respectively, recorded for a selectively leucine and valine methyl protonated α-spectrin SH3 sample. (B) Spin echo evolution curves under OffMAS for residues V53*γ*1 (black) and V23*γ*1 (red), respectively. Error bars are smaller than the size of the symbols. 1D traces for V53*γ*1 are plotted at echo delays of 200 μs, 5.2 ms, 14.8 ms, respectively.*



***Figure 3:*** *Correlation of methyl order parameters measured using the OffMAS approach and REDOR dephasing (Asami and Reif 2013). The OffMAS experiment was carried out using 𝛥𝜃𝑅𝐿 = −0.12°, employing 55 kHz MAS. The spectrum was recorded at an external field strength B0 of 18.8 T (800 MHz for 1H).*

The dipolar coupling values obtained from OffMAS and REDOR methods are compared in Figure 3. OffMAS and REDOR experiments are recorded under slightly different conditions: The REDOR experiment was acquired using 2H decoupling during the 13C *t*1 evolution period whereas no 2H decoupling was employed in the OffMAS experiment. This leads to larger linewidths in the 13C dimensions in the OffMAS spectra as the 2H,13C scalar couplings evolve during the indirect dimension. INEPT based magnetisation transfer schemes were employed both for OffMAS and REDOR experiments. We find that both methods yield very similar order parameters. In addition to dipole anisotropy information, the experiments contain information on the asymmetry parameter. This has been shown previously using REDOR-type experiments (Schanda et al. 2011a; Schanda et al. 2011b). In principle, this information is contained as well in the OffMAS dipolar oscillations. Work into this direction is currently in progress in our laboratory.

**Conclusions**

Taken together, we have shown that slight mis-setting of the spinning angle in a high-resolution MAS solid-state NMR experiment allows to quantify methyl order parameters in a selectively methyl protonated microcrystalline protein sample. We believe that this kind of experiment will be useful for the quantification of dynamics in the solid-state at very fast spinning, where even short high-power pulses occupy a significant fraction of one rotor cycle. High accuracy in quantifying order parameters using the off-MAS method requires long coherence life times. A reduction of the error bars can be achieved by employing perdeuteration in combination with ultrafast-MAS.

**Materials and Methods**

**Sample Preparation**

Microcrystalline α-spectrin SH3 was prepared as described previously (Chevelkov et al. 2006). Methyl labelling using α-ketoisovalerate was achieved as described by Agarwal et al. (Agarwal et al. 2008).

**NMR Spectroscopy**

NMR experimentswere carried out using a Bruker Avance 3 spectrometer operating at a 1H Larmor frequency of 800 MHz. The MAS rotation frequency was set to 55 kHz. 1H and 13C hard pulses were applied using a RF field strength of ω1H/2π = 166.7 kHz, ω13C/2π = 78.1 kHz RF fields, respectively. The sample temperature was maintained at about 10 °C with a Bruker BCU-X unit, setting the cooling gas flow rate to 1200 litres per hour. 13 spin echo delays were measured with 16 scans each employing a recycling delay of 1.2s. The maximum echo delay was set to 32 ms. For each 2D slice, the acquisition time in the indirect dimension was set to 36.4 ms.

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